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Combining biotechnology and evolution for understanding the mechanisms of pollinator attraction

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Many flowering plants rely on pollinators for their reproductive success. Plant-pollinator interactions usually depend on a complex combination of traits based on a fine-tuned biosynthetic machinery, with many structural and regulatory genes involved. Yet, the physiological mechanisms in plants are the product of evolutionary processes. While evolution has been modifying flowers through millions of years, it is also a rapid process that can change plant traits within few generations. Here we discuss both mechanistic and evolutionary aspects of pollinator attraction. We also propose how latest advances in biotechnology and evolutionary studies, and their combination, will improve the elucidation of molecular mechanisms and evolutionary dynamics of pollinator attraction in changing environments.

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Introduction

Most flowering plants rely on pollinators for their reproductive success and have evolved a diversity of strategies to attract them. Past and recent evolution shaped the attractiveness of plants, leading to complex combinations of floral traits associated with pollinator attraction. These multiple traits can be genetically correlated, impacting their rate of evolution [1]. For example, as a response to altered pollinator communities, linked traits may evolve faster due to their simpler genetic architecture. The ongoing natural and anthropogenic environmental changes impact plant-pollinator associations in many ways, for example, through decline of specific pollinator species [2] or desynchronization of plant flowering and pollinator activity [3,4] reducing plant reproductive success. In this context, plants have to adapt rapidly to attract novel pollinators [5]. Adaptive processes have typically been studied in selfing model plant species such as *Arabidopsis* or *Boechera* in the context of climate changes [5,6]. However, few studies addressed the adaptive potential of outcrossing plant species to changing pollinator niches, despite the dependence of a large majority of angiosperms on pollinators. It is therefore essential to better understand the adaptive potential of plants, in regard to their ability to attract (novel) pollinators [7], as well as the underlying genetic basis and molecular mechanisms. Here, we briefly review the current knowledge and identify gaps and future perspectives on both mechanistic and evolutionary aspects of pollinator attraction. We also discuss how a merge of the latest breakthroughs in biotechnology and evolution will advance a comprehensive study of plant attractiveness to pollinators.

Floral attractiveness traits and their underlying molecular pathways

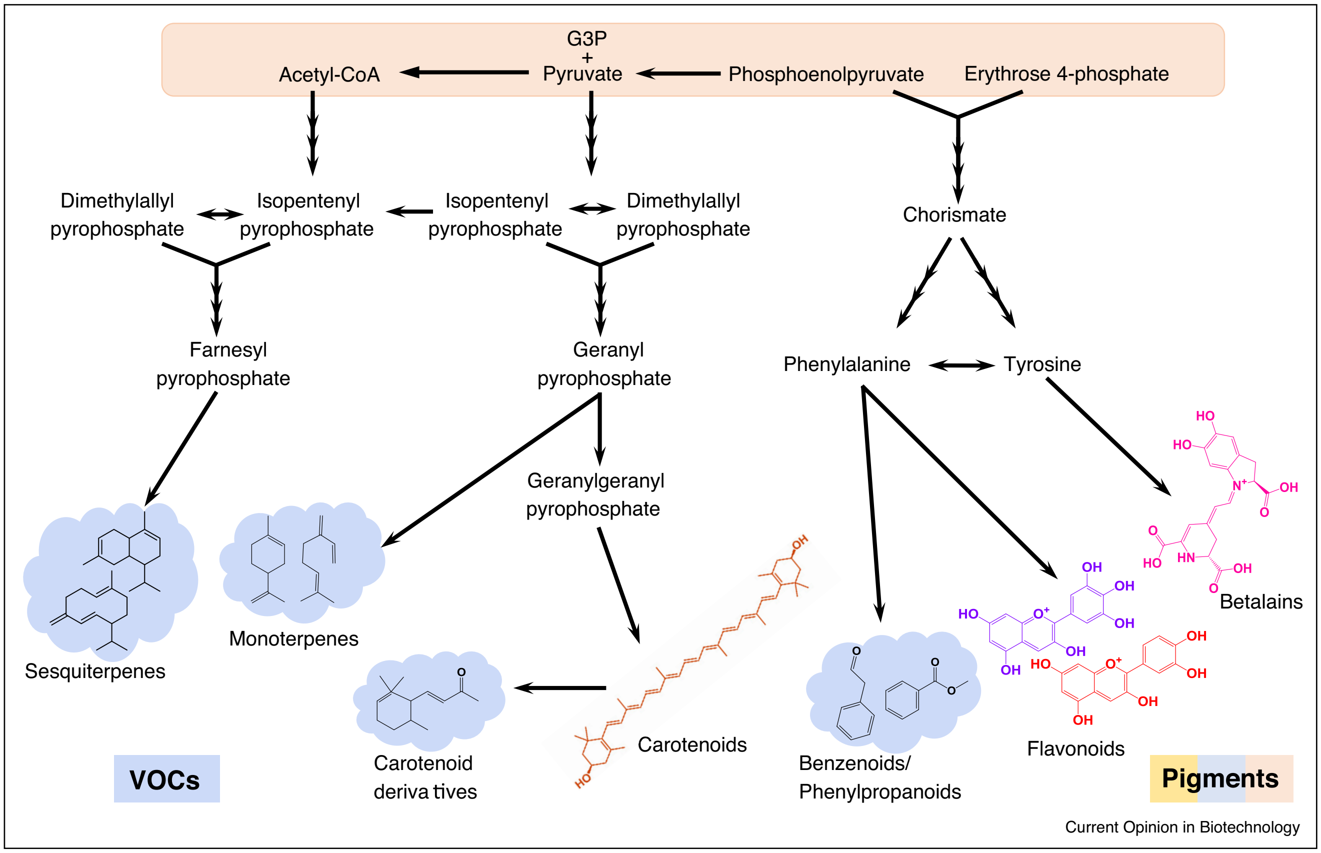
For successful pollination, plants have to guide pollinators towards their flowers by providing signals (scent, color, size, CO2, temperature, etc.), and usually reward them with nectar, pollen, oil, fragrance, or resin [8]. Pollinators are attracted to specific flowers because of their preferences for signals that can be innate or learned depending on pollinator species. Specialized pollinators (i.e. a pollinator visiting one or few plant species) are often particularly sensitive to specific signals [9] leading to convergent evolution of signals in unrelated species, called pollination syndromes [10]. For instance, the genus *Petunia* is well known for its different pollination syndromes among sister species [11,12]. Generalist pollinators (i.e. those visiting several plant species), however, typically learn to associate a signal with a reward, thus attraction is usually based on learning [13]. Such associative learning, in connection with plant reproductive traits, may select for the evolution of honest signals (i.e. those that reliably indicate rewards) in plants [14].

Within the range of floral signals, odors and colors are probably the most studied due to their important roles in many plant-pollinator interactions. To date, pathways leading to pigment formation have been largely elucidated. Three major groups of pigments contributing to flower colors include (1) flavonoids, mainly anthocyanins, responsible for orange, red, purple, and blue coloring, (2) carotenoids providing red and yellow pigmentation, and (3) betalains yielding red/violet or yellow/orange color in flowers of the Caryophyllales (Figure 1) [15,16]. In contrast to pigments, less is known about the biosynthesis of floral volatiles. Despite their extraordinary diversity, floral volatile organic compounds (VOCs) are divided into three major classes including terpenoids (monoterpenes and sesquiterpenes), aromatics (phenylpropanoids/benzenoids), and fatty acid derivatives [17,18]. In the last two decades, a large number of genes encoding enzymes responsible for the most widespread compounds have been characterized, however, most scent biosynthetic genes still remain unknown [19]. These recent discoveries have built a foundation for investigating the role of individual volatiles in pollinator attraction [16,17,20], however, the effects of altered volatiles on pollinator attraction has rarely been studied.

Evolutionary aspects of correlated traits

Analysis of the past and recent evolutionary processes can teach us about the adaptive potential of plants to ongoing changes. Plant attractiveness involves a combination of traits that may be either independent, or associated with linked genetic regions [21,22]. A good example are honest signals (Figure 2a), which are signals quantitatively associated with reward amounts (Figure 2b). Such signals may be based on linkage, driven by selection for honesty, or on pleiotropy (see below & Figure 2c) [13,23]. Genetic correlation can lead to floral integration (i.e. correlation among floral traits), as a consequence of past selection for covariance of traits. Within floral scent bouquets, overall phenotypic integration was shown to have low consistency in the component's ratios [24], however, strong

Figure 1



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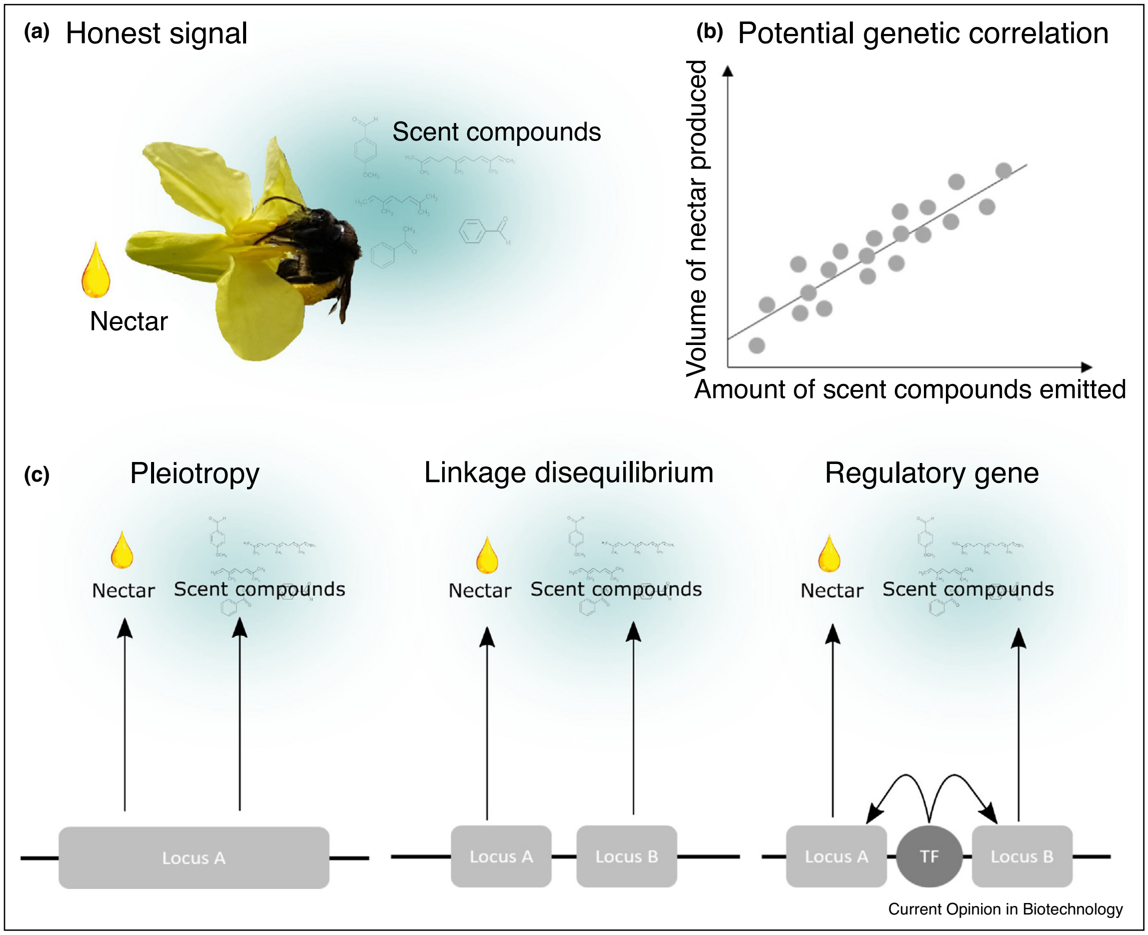
Biochemical interconnection between volatile scent compounds and floral pigments.

Volatile sesquiterpenes (germacrene D and cadinene), monoterpenes (limonene and myrcene), benzenoid/phenylpropanoids (methylbenzoate and phenylacetaldehyde), and carotenoid derivatives (beta-ionone) are shown on blue cloud as background. Shown pigments include carotenoids (zeaxanthin), flavonoids (cyanidin and delphinidin), and betalains (betacyanin). Stacked arrows depict multiple enzymatic steps in the pathway. Compounds in the top light red box are primary metabolites that serve as precursors for VOC and pigment biosynthesis. VOC, volatile organic compounds.

Figure 2



Hypothetical causes of genetic correlation for honest signal.
(a) Example of honest signal in *Brassica incana*. **(b)** Potential genetic correlation between scent compounds and nectar. **(c)** Illustration of causes of potential genetic correlation between floral traits; the pleiotropy that is, one genetic variant controls the phenotypic traits variations, the linkage disequilibrium that is, two genetic variants are linked controlling different phenotypic traits, and the regulatory gene that is, a transcription factor (TF) controls the gene expressions of two loci involved in the variation of different phenotypic traits.

correlation among groups of scent compounds has been detected [25,26•], leading sometimes to a relatively simple genetic architecture involving only few quantitative trait loci [27]. Moreover, there is strong evidence for the existence of pleiotropic links between volatile compounds and color pigments as they often derive from common biosynthetic precursors (Figure 1) [16,19]. Multiple studies showed that white flowers of different plant species emit higher levels of benzenoid/phenylpropanoid scent compounds than their colored counterparts (reviewed in [28]). Color-odor interconnections were also confirmed by metabolic engineering where the suppression of pigment production in transgenic plants resulted in an increase in scent emission [29], and *vice versa*, the reduction of scent compounds lead to an increase of color pigments [30,31]. These insights into the link between color and scent suggest that biosynthetic pleiotropy may often be important, thus slowing down or prohibiting the independent evolution of both signals. A fascinating example of linkage between floral traits comes from the crucifer genus *Moricandia*, showing seasonal phenotypic plasticity in flower types, with large, cross shaped lilac flowers produced in spring, and small, round shaped white flowers in summer [32••]. Such unique plasticity based on differential gene expression offers novel insights into the function and evolution of correlated floral traits.

Genetic correlation of multiple traits involved in plant attractiveness can facilitate or constrain plant adaptation [1]. An advantageous genetic correlation, causing simultaneous trait changes and reducing antagonist effects among traits, may allow rapid answers to pollinator changes [11,21,33]. Such correlations may be caused by linkage disequilibrium (i.e. correlation of the linked genetic variants), real pleiotropy (i.e. one genetic variant involves multiple traits), or regulatory genes (i.e.

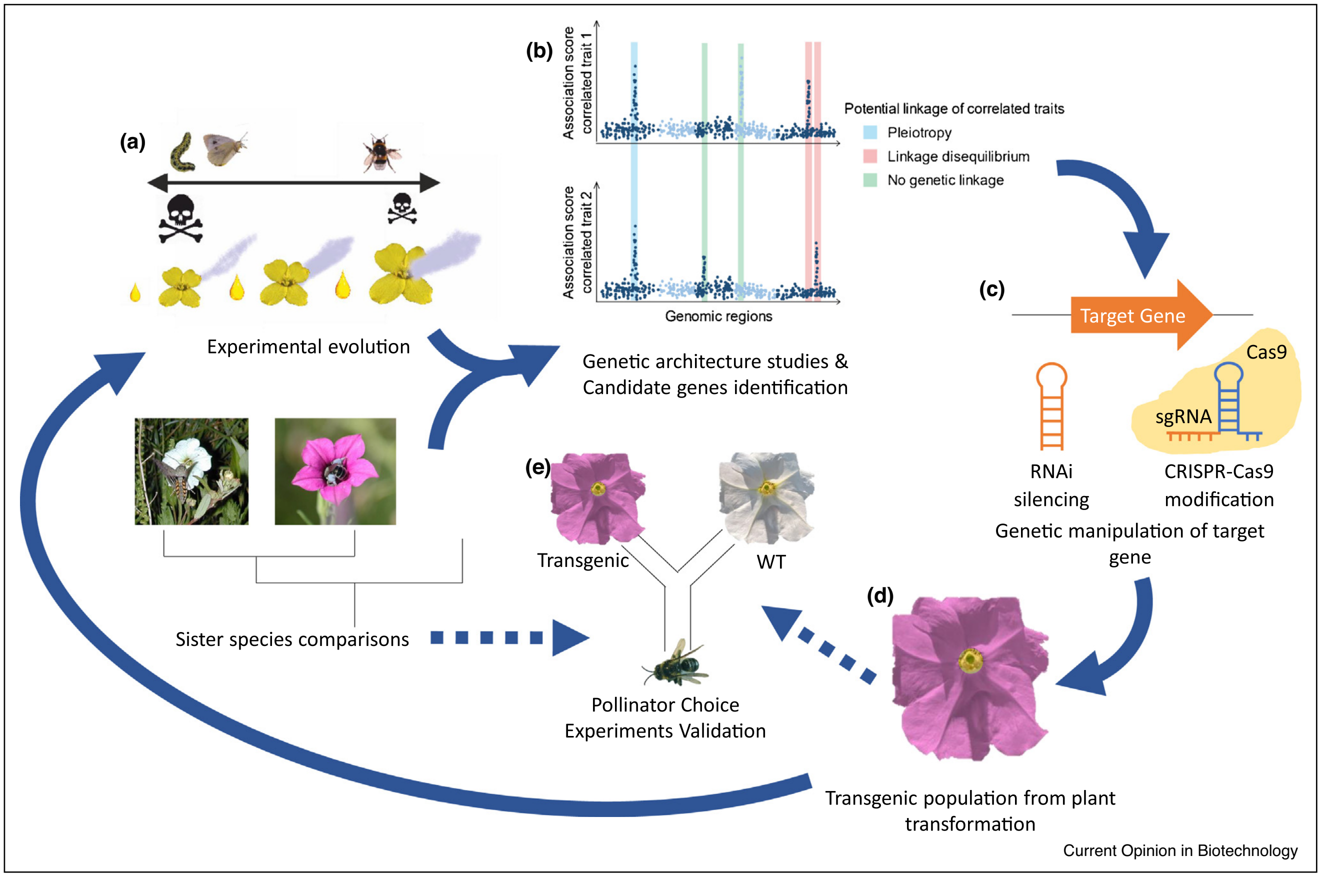
transcription factors controlling two genes impacting two different traits), with different implications on evolutionary trajectories and rates of change (Figure 2c) [21,34]. While it was difficult to distinguish among the causes of genetic linkage up till a decade ago [35], the development of new sequencing technologies with higher resolution, as well as powerful analytical tools (e.g. Genome-Wide Association: GWA) opens the door to a deeper understanding of the genetic architecture underlying linked phenotypic traits (Figure 3).

Studies identifying the genetic basis of traits associated with attractiveness, linkage of traits, and plasticity are in their infancy and need further investigation. Better characterization of the genetic architecture of correlated traits on a whole genome level (identity of genes, number of genes, effect of genes on phenotypic trait variation, gene interactions, etc.) is essential in the understanding of rapid adaptive evolution of attractiveness in plants and a complementary approach for elucidating the underlying molecular pathways and their overlap. In addition, the combination of genomic studies with functional biotechnology offers novel insights by combining genetic architecture with gene function, thus providing an invaluable link to the phenotype.

Metabolic engineering to unravel the mechanisms of pollinator attraction

Metabolic engineering has been already successfully used to modify floral color and fragrance to increase aesthetic and commercial values of ornamentals [15]. As such, the assessments of attractiveness of manipulated traits were

Figure 3



Integration of evolutionary and biotechnological approaches to understand the mechanisms of pollinator attraction.
(**a**) Two experiments designed at intraspecies level with experimental evolution (upper panel) over generations and at interspecies level comparing sister species (bottom panel) allowing to track the phenotypic and genomic changes. Using herbivores as selective pressure in experimental evolution, a decrease of floral scent compounds and nectar volume, and an increase of toxic compounds are expected. Conversely, pollinators may drive an increase of VOCs and nectar volume, as well as a decrease of toxic compounds. (**b**) The Genome-Wide Association (GWA) mapping performed on two different traits, for instance scent compounds and nectar volume, allows to identify genomic regions associated with phenotypic trait variations. Represented by Manhattan plots, the genomic architecture can be compared and the causes of potential correlated traits (pleiotropy, linkage disequilibrium, regulatory gene) can be highlighted. Once a gene has been identified by GWA, its expression can be modified via RNAi silencing or CRISPR-Cas9 gene editing (**c**) followed by generation of mutants (**d**). For functional gene validation, mutants can be compared to wild type in pollinator choice experiments (**e**). Knowledge gained can be further applied by fine tuning evolutionary experiments and repeating the different steps (a)–(e).

limited to humans' perception and the effects of these perturbations on pollinator attraction were only rarely studied. The contribution of color to attraction of pollinators was investigated mainly without applying biotechnological approaches. Indeed, field tests with near-isogenic *Antirrhinum majus* lines with red, pale pink, ivory and white flowers revealed that flowers with full red color were more preferred by bumblebees, natural pollinators of snapdragon flowers [36]. Moreover, in contrast to palely pigmented or white lines, bumblebees visited significantly more flowers per plant in the red-flowered plants. Interestingly, expression of *Venosa* gene encoding the R2R3 MYB transcription factor in a pale or acyanic snapdragon background led to formation of pigment stripes associated with veins and increased flower attractiveness to a level comparable with that of the full-red flower [36]. Unfortunately, scent profiles and their contribution to pollinator attraction were not examined in this study.

The first investigation of the consequences of metabolic engineering of floral VOCs on pollinator visitation was performed in transgenic tobacco *Nicotiana attenuata* plants. Silencing genes in the targeted biosynthetic pathways reduced the levels of benzyl acetone and nicotine, the most abundant attractant and repellent compounds, respectively, present in both tobacco flower bouquet and nectar [37]. Monitoring the activity of floral visitors in their native habitat revealed that plants lacking benzyl acetone (both with and without nicotine) received fewer visits from hawkmoths and hummingbirds than flowers emitting this volatile. Reduction in nicotine, which comprises 25% of the nectar constituents, increased consumption of nectar by the native community of flower visitors, suggesting that a single defense compound can profoundly influence nectar removal [37,38]. The obtained results also imply that by regulating nicotine concentration in flowers plants can decrease nectaring time and nectar volume removal and increase the number of flower visitors, while keeping their nectar volume small [37,38], and promote outcrossing rate in *N. attenuata* by triggering hummingbirds to visit more flowers in search for low-nicotine flowers [39]. Changes in more than one volatile compound can also influence pollinator behavior. Indeed, ectopic expression of the *Arabidopsis* Production of Anthocyanin Pigment 1 (PAP1) transcription factor in rose flowers enhanced production of phenylpropanoid and terpene volatiles, which was easily distinguished by honeybees [40], the native pollinators of some wild rose species [41].

In general, floral scent is a complex mixture of volatile compounds containing in most species between 20 and 60 different volatiles [24,42]. To date little is known about the contribution of individual compounds to plant-insect interactions [but see: 9,43]. However, utilization of various *Petunia* transgenic lines with RNAi silenced ability to produce different flower volatiles in field experiments allowed elucidation of the distinct roles of individual volatile compounds in attraction of mutualists and deterrence of antagonists [44]. Indeed, within the *Petunia* scent profile, isoeugenol and benzyl benzoate specifically control infestation rate by florivores, whereas methyl benzoate is involved in pollinator attraction. As pollinators and florivores use the same visual and olfactory landscape to locate the host, plants have to balance attracting and deterring functions of floral volatiles. In addition to having deterrent compounds in the scent bouquet [44], flowers can protect their reproductive organs via inter-organ aerial transport of volatiles via natural fumigation [45•]. For example, in *Petunia* flowers tube-produced sesquiterpenes are released inside the buds and accumulate in the stigma, thus not only affecting bacterial growth on stigma but are also supporting optimal pistil growth and seed yield.

Perspectives

A powerful way to understand the evolutionary potential and the constraints involved in plant attractiveness are evolution experiments (Figure 3). When combined with high-throughput sequencing, this approach allows the identification of the genomic changes involved in phenotypic evolution and their underlying molecular mechanisms [46]. This method, traditionally developed in microorganisms (*Escherichia coli*, *Pseudomonas*, yeast) and insects with short generation time (*Drosophila*) has recently been also applied to plants [5,47]. The power of experimental evolution is the possibility of precise manipulation of selective factors, and the observation of real-time phenotypic and genomic evolutionary changes as a consequence. Using semi-natural selective factors (e.g. pollinators) [5], or artificial selection [48], this approach allows the study of the evolution of specific traits or trait combinations such as those involved in honest signaling or pollination syndromes, the (in)dependency of their evolution, and their plasticity [49•]. The recent improvement of gene editing technologies (e.g. CRISPR-Cas9), and the premises of polygenic engineering allowing for multi-gene knockouts [50] are promising tools for future generation of mutants for functional validation of genes involved in complex and correlated traits. Based on these validated genes, new genotypes can be selected for experimentally evolving populations. For example, novel nectarless "cheater" genotypes could be added to otherwise honestly signaling plant populations, to study the conditions under which they may stabilize or even spread. Alternatively, novel color and scent genotypes would provide the opportunity to test the impact of more attractive individuals on the stability of plant-pollinator networks. The genus *Petunia* or *Nicotiana* may be good candidates for such approaches due to the rich data on molecular mechanisms and availability of gene transformation. Such novel approaches will provide insights into functions of floral traits as well as into the evolvability of pollinator-attracting traits and trait combinations, essential for improving the ecosystem management and the breeding of crop species more attractive to pollinators.

Conflict of interest statement

Nothing to declare.

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